3-EPI-20-HYDROXYECDYSONE FROM MECONIUM OF THE TOBACCO HORNWORM

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ABSTRACT

A new ecdysteroid, 3-epi-20-hydroxyecdysone, with biological activity 1/10 to 1/15 that of α-ecdysone has been isolated and identified from the meconium of the tobacco hornworm. The possible role or function of this steroid as an inactivation product and/or regulator of molting hormone titer is discussed.

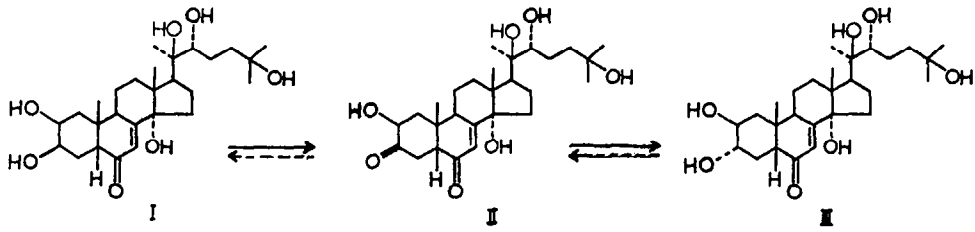
Thus far, there are four known insect molting hormones, α-ecdysone, 20-hydroxyecdysone, 20,26-dihydroxyecdysone, and 26-hydroxyecdysone; also, presently, Manduca sexta (L.) is the only insect from which all four of these ecdysones have been isolated and identified (1, 2, 3). However, not until the identification of 26-hydroxyecdysone as the predominant ecdysone from the developing egg of the tobacco hornworm was there any indication that there are qualitative and quantitative differences in the molting hormones at the different developmental stages of this insect (3). Although α-ecdysone serves as a precursor for the insect ecdysones, until the isolation of 26-hydroxyecdysone, 20-hydroxyecdysone had been the major molting hormone to be isolated from the tobacco hornworm or its excretory products (4). In our attempts to better understand the biosynthesis, metabolism, and inactivation of the ecdysones, we have continued our search for new ecdysones, precursors, and metabolites that are present at different stages of insect development. In this paper, we report on the isolation and

identification of 3-epi-20-hydroxyecdysone (III) from the meconium of the tobacco hornworm.

The meconium fluid, obtained from tobacco hornworms of mixed sexes shortly before eclosion as previously described (5), was collected into 50 ml glass-stoppered centrifuge tubes containing 9 ml of butanol and then stored at 0°. When the volume of the fluid in a series of 6-20 tubes reached 45 ml, the tubes were thoroughly shaken and centrifuged to separate the phases. The butanol phase was removed from each tube, the lower layer was reextracted twice again with 9 ml of butanol, and the butanol extracts were combined and kept in a glass container at 0°. When the butanol extracts from about 3 liter-equivalents of meconium had been accumulated, they were further processed as described previously (6). Approximately 12.4 g of crude extractives from a total of 14.5 liters of meconium fluid was chromatographed on Woelm Neutral Alumina (Grade I plus 20% water) with methanol as the eluting solvent (7). The residue (4.2 g) obtained from the methanol eluant was further purified by chromatography on a silicic acid column with increasing percentages of methanol in benzene (6). The biologically active benzene-methanol (90:10) fraction (261 mg) was subjected to 50 transfers in a counter current distribution system of cyclohexane-butanol-water (5:5:10) with 100 ml each of upper and lower phase and was then separated into two fractions (tubes 11-22 and tubes 33-45).

The material from tubes 11-22 (25 mg) was further purified by chromatography on a silicic acid column. The 90:10 benzene-methanol fraction, when analyzed by TLC showed two spots that were not completely resolved. When the entire fraction was subjected to preparative TLC on a 20 x 20-cm

plate and developed to length of plate in the solvent system of chloroform-ethanol (4:1), it separated into two zones. The material from each zone was further purified by column chromatography on silicic acid. Chromatography of the material from the upper zone (8.6 mg), and crystallization from ethyl acetate-methanol of the material eluted from the column with benzene-methanol (90:10) gave 3.6 mg of crystals, m.p. 225-229° with slight decomposition. The steroid exhibited a R_f of 0.17 (20-hydroxyecdysone, R_f 0.14) and ultraviolet absorption maximum at 245 nm (methanol), ϵ 10,800 (8), and was 1/10 to 1/15 as active as α-ecdysone in the house fly molting hormone assay (9). Its NMR spectra, recorded at 60 megacycle in deuterated pyridine, exhibited methyl resonances at δ 1.20 (18-H), 1.08 (19-H), 1.58 (21-H), 1.38 (26- and 27-H), which are identical to those of 20-hydroxyecdysone and suggested that the environment of the methyl groups were similar. The mass spectrum fragmentation pattern showed a M^+ peak at m/e 480 and was similar to that of 20-hydroxyecdysone in the high mass range (m/e 480-250), though it differed in the region of m/e 250-100; also, like 20-hydroxyecdysone, it exhibited its base peak at m/e 99 with the second largest peak occurring at m/e 81. The reaction of the compound with acetone in the presence of p-toluenesulfonic acid yielded a monoacetonide; 20-hydroxyecdysone, under similar conditions, gave predominantly a diacetonide. If the compound has a 2,3-diol system, then the formation of a monoacetonide indicates a trans-diol at the 2,3-position, and further suggests that the acetonide formed was the 20,22-monoacetonide and that this ecdysone differs from 20-hydroxyecdysone in that it has a 2β,3α- or 2α,3β-diol system. Support for the assigned 2β,3α-diol structure is the following: The presence



of a 2α-hydroxyl group would have caused a slight, though noticeable down field chemical shift in the C-19 methyl signal from that of 20-hydroxyecdysone, and this did not occur. Both the synthetic 2β,3α,14α-trihydroxy-5β-cholest-7-en-6-one (IV), and this new ecdysone migrated slightly faster than their corresponding 3β-epimers by TLC in a 4:1 chloroform-ethanol solvent system. The isomerization of the new ecdysone with 1% potassium carbonate in 90% methanol at 50° gave the corresponding more polar 5α-epimer. Compound IV is less polar than its corresponding 5α-epimer. On the other hand, 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one and 20-hydroxyecdysone are more polar by TLC than their corresponding 5α-epimers. Finally, we have enzymatically transformed α-ecdysone to a less polar pentahydroxy steroid isomer that does not form an acetonide derivative (10). Since we can also enzymatically convert 20-hydroxyecdysone to a corresponding less polar hexahydroxy steroid that behaves chromatographically by TLC and high-pressure liquid-solid chromatography (HPLSC) on Corasil II (11) as the steroid isolated from the meconium, we

conclude that this hexahydroxy steroid (III) is 2β,3α,14α,20R,22R,25-hexahydroxy-5β-cholest-7-en-6-one (3-epi-20-hydroxyecdysone).

The substantially pure but not crystalline compound (1.7 mg), obtained from the lower zone and which exhibited a R_f similar to that of 20-hydroxyecdysone (R_f 0.14), was characterized as 20-hydroxyecdysone by biological activity (quantitated by UV analyses), TLC, NMR, and mass spectral analyses.

Similarly, the biologically active material in counter current distribution tubes 33-45 (160 mg) was partially purified to a final mass of 6.4 mg as in the purification of 3-epi-20-hydroxyecdysone. Although we obtained from this fraction, in considerably less quantity, a compound (R_f 0.27) that was chromatographically less polar than α-ecdysone (R_f 0.23) by TLC and HPLSC and that could possibly be the 3-epiecdysone, we have not definitely established its identity.

The first sterol sulfate conjugates to be isolated from an insect were isolated from the meconium of the tobacco hornworm, and the relatively large percentage of the phytosterol found as sulfate conjugates in the meconium suggested selective elimination of these phytosterols into the meconium during pupal-adult development (5). Since the meconium serves as a depository for waste, metabolized products, and excess material, these ecdysones could very well represent inactivation products of the ecdysones. The low biological activity, 1/10 to 1/15 that of α-ecdysone, lends some support to this conclusion. However, the fact that a detectable quantity of 20-hydroxyecdysone, an active molting hormone, was also present in the meconium suggests that each of the ecdysones could have specific function(s).

Although 3-dehydro-20-hydroxyecdysone (II) has not as yet been isolated from the tobacco hornworm, this steroid is probably the intermediate involved in the conversion of 20-hydroxyecdysone to its 3α-epimer and this may be a reversible reaction as shown in the scheme. If the 3-dehydroecdysones intermediates that have been isolated from *in vitro* and *in vivo* systems (12, 13) also have specific functions in insects, their conversion to the 3α-epimers could be a means of inactivation of these dehydro compounds. The postulated interconversion of the ecdysones and the 3α-epimers could also be a means of regulating the titer of the ecdysones.

Karlson and Koolman (13) recently concluded from *in vivo* studies with white prepupae of *Calliphora vicina* Robineau-Desvoidy that the 3-dehydroecdysone is a side reaction product of α-ecdysone which is rapidly reconverted to α-ecdysone. The 3α-epimers are not readily separated by TLC from the 3β-epimers and since the conversion of the 3-dehydro-ecdysones, whether to the ecdysones or their 3α-epimers, may be of considerable significance in further understanding the roles and actions of the ecdysones, it is important that the products obtained from the *in vivo* reduction of 3-dehydroecdysone be unequivocally identified.

In an earlier paper, we reported the isolation from 7 day-old tobacco hornworms, during pupal-adult development, of an unidentified compound that was slightly less polar than 20,26-dihydroxyecdysone (2). This compound could well be the 3α-epimer of 20,26-dihydroxyecdysone. Other publications report unidentified compounds that may be the 3α-epimer of 20-hydroxyecdysone: Miyazaki *et al.* (14) detected an unknown ecdysone in 2 day-old pupae of the silkworm *Bombyx mori* (L.), that was

slightly less polar than 20-hydroxyecdysone and was present in a greater quantity in older pupae. Similarly, radiolabeled α-ecdysone is converted by larvae of Chironomus tentans (L.) to 20-hydroxyecdysone which is then further converted into a metabolite of unknown structure that migrates slightly in front of α-ecdysone by TLC and with 20-hydroxyecdysone by reverse phase chromatography (15). If the unidentified ecdysone from these two species of insects is also eventually identified as the 3α-epimer of 20-hydroxyecdysone, then the 3-epiecdysones may be as common to insects as are α-ecdysone and 20-hydroxyecdysone. Additional research concerned with the quantitative differences in the titers of these epimers and with the ecdysones at different developmental stages of insects, and with the enzyme(s) involved in this biotransformation is required before their function and action can be understood.

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7. Mention of a commercial or proprietary product in this paper does not constitute an endorsement of this product by the U. S. Department of Agriculture.

8. It is likely that the melting point and extinction coefficient of this compound highly purified would be considerably higher since most ecdysones melt at temperatures between 235 and 260° and have

an ϵ about 12,000. The melting point and ϵ of the synthetic 2β,3α,14α-trihydroxy-5β-cholest-7-en-6-one are 251° and 12,000 respectively.

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TRIVIAL AND IUPAC EQUIVALENT NAMES

α-Ecdysone = 2β,3β,14α,22R,25-Pentahydroxy-5β-cholest-7-en-6-one

20-Hydroxyecdysone = 2β,3β,14α,20R,22R,25-Hexahydroxy-5β-cholest-7-en-6-one

3-Epi-20-hydroxyecdysone = 2β,3α,14α,20R,22R,25-Hexahydroxy-5β-cholest-7-en-6-one

3-Dehydro-20-hydroxyecdysone = 2β,14α,20R,22R,25-Pentahydroxy-5β-cholest-7-en-3,6-dione

26-Hydroxyecdysone = 2β,3β,14α,22R,25,26-Hexahydroxy-5β-cholest-7-en-6-one

20,26-Dihydroxyecdysone = 2β,3β,14α,20R,25,26-Heptahydroxy-5β-cholest-7-en-6-one